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Heterologous Expression of a Hyperthermophilic α-Amylase in Xanthan Gum Producing *Xanthomonas campestris* Cells

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Abstract A hyperthermophilic α-amylase encoding gene from *Pyrococcus woesei* was transferred and expressed in *Xanthomonas campestris* ATCC 13951. The heterologous α-amylase activity was detected in the intracellular fraction of *X. campestris* and presented similar thermostability and catalytic properties with the native *P. woesei* enzyme. The recombinant α-amylase was found to be stable at 90 °C for 4 h and within the same period it retained more than 50% of its initial activity at 110 °C. Furthermore, *X. campestris* transformants produced similar levels of recombinant α-amylase activity regardless of the carbon source present in the growth medium, whereas the native *X. campestris* α-amylase production was highly dependent on starch availability and it was suppressed in the presence of glucose or other reducing sugars. On the other hand, xanthan gum yield, which appeared to be similar for both wild type and recombinant *X. campestris* strains, was enhanced at higher starch or glucose concentrations. Evidence presented in this study supports that *X. campestris* is a promising cell factory for the co-production of recombinant hyperthermophilic α-amylase and xanthan gum.

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Introduction

Xanthomonas campestris is a Gram-negative bacterium that produces high yields of an extracellular polysaccharide, xanthan gum, which is used as thickener, emulsifier, and stabilizer in oil drilling, textile, and food industries due to its high viscosity and pseudoelasticity [1]. *X. campestris* is also known to produce a variety of enzymes including a periplasmic α-amylase the gene of which was cloned and expressed in *Escherichia coli* [2].

Bacterial α-amylases are widely used in the industry for starch hydrolysis [3]. Due to their thermostability, enzymes isolated from hyperthermophilic microorganisms present a great biotechnological interest for the structural basis of their thermostability. Recently, α-amylases isolated from hyperthermophilic microorganisms have attracted considerable biotechnological interest, as starch is solubilized at temperatures as high as 100 °C and the potential use of thermostable α-amylases is more suitable for this process [4, 5]. However, because of inherent difficulties of cultivation of hyperthermophiles, essentially due to the high cost for heating the fermentors and the low cellular mass yield, expression of hyperthermophilic enzymes in mesophilic hosts without losing their catalytic properties is of primary importance.

In the present work, we describe the expression in *X. campestris* of the *Pyrococcus woesei* α-amylase, a hyperthermophilic enzyme classified in family 13 of the glucosyl hydrolase superfamily [6]. The *P. woesei* α-amylase gene was previously transferred and expressed successfully in *E. coli* and *Halomonas elongata* where it conferred its hyperthermophilic properties [7]. The expression of such a heat-stable enzyme in *X. campestris*, and the investigation of the *X. campestris* transformants for their potential co-production of xanthan gum and α-amylase under conditions of maximum production of xanthan gum, is addressed for the first time.

Materials and Methods

Strains, Plasmids, and Growth Conditions

Escherichia coli DH5α (BRL, Gaithersbourg, MD), which was used as a host for subcloning and maintaining recombinant plasmids, was grown in LB medium by standard methodology [8]. *X. campestris* ATCC13951 (wild type) and recombinant strains were grown in LB plus 0.2% glucose (LBG) or in minimal M9 medium containing various concentrations of glucose or starch. Solid media were obtained by adding 2% (*w*/*v*) agar. The recombinant plasmids pCPP30 (Bauer, personal communication, 9) and pHS15/P_{wo}*amy* [7] were used for the construction of pCPP30/P_{wo}*amy* and the isolation of α-amylase gene, respectively. For genetic selection or plasmid maintenance, tetracycline 20 μg ml^{-1} was used. Plasmid stability was estimated as described before [7]. Lactose or isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 25 and 1 mM, respectively, was added in the cultures when needed, for the induction of *amy* under P_{lac}, at culture $OD_{600\ nm}$ ~ 0.5.

DNA Isolation, Manipulation, and Transformation

Plasmid DNA extraction from *E. coli* strains, DNA restriction, ligation, *E. coli* transformation, and Southern blotting were carried out by standard methodology [8].

Plasmid DNA from *X. campestris* wild type and transformed strains was isolated by using the Promega Wizard Plus Kit (Catalogue No A1330). DNA bands from agarose gels were purified by silica [10]. Hybridizations were performed under high-stringency conditions with a digoxigenin-11-dUTP labelled probe using the Boehringer Mannheim non-radioactive DNA labelling and detection kit (Catalogue No 1093657) according to the manufacturer's instructions. Competent *X. campestris* cells for electroporation were prepared and recovered as previously described [9–11].

Measurement of α-Amylase Activity

E. coli and/or *X. campestris* strains harboring the α-amylase gene (as well as the strains carrying the vector pHS15 as negative control) were grown up to the late exponential phase in 150 ml cultures. The cells were harvested (3,000 g, 20 min), washed with 0.9% (*w*/*v*) NaCl and resuspended in 5 ml of 50 mM sodium acetate buffer, pH 5.5. Lysozyme (1 mg ml^{-1}) for 45 min and sonication (5×20 s, 0.5 cycle, 50% amplitude) were used for cell lysis. The mixture was centrifuged at 3,000 ×*g* for 10 min at 4°C and this supernatant was used as the initial enzyme preparation after the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). α-Amylase activity was determined at 95°C according to the method already described [7]. One unit of α-amylase is defined as the enzyme activity required to hydrolyze 1 mg starch in 1 min, as determined by the absorbance decrease in the starch–iodine reaction.

X. campestris wild type cells were removed from the culture broth by centrifugation at 3,000 ×*g* for 20 min at 4°C and the resulting cell free supernatant was fractionated with solid ammonium sulfate. The precipitate at 80% saturation of ammonium sulfate was collected by centrifugation and suspended in minimum amount of 50 mM sodium acetate buffer, pH 5.5. The natural *X. campestris* α-amylase activity was determined at 37°C.

Total protein was estimated by the Bradford method [12].

Determination of Xanthan Gum

Xanthan gum was determined as previously described [13].

Statistical Analysis

All experiments were performed in triplicate and the data presented in this work are the mean value of three different experiments. Standard error was calculated by standard statistics.

Results

Transfer and Expression of the *P. woesei* α-Amylase Gene in *X. campestris*

In a previous work, Koch et al. [14] purified and characterized a heat-stable α-amylase from the cell free culture media of *P. woesei*. Recently, the hyperthermophilic α-amylase gene was cloned from *P. woesei* to the moderate halophile *H. elongata*, which lacks native α-amylase activity, using the shuttle vector pHS15/P_{wo}*amy* [7].

The α-amylase gene of the hyperthermophilic archaeon *Pyrococcus woesei* was isolated from plasmid pHS15/P_{wo}*amy* [7] by *Pst*I digestion and subcloned in pCPP30 MCS under

the control of the P_{lac} promoter, resulting in recombinant plasmid pCPP30/P_{wo}*amy* (Fig. 1). Vector pCPP30 (Bauer, personal communication; 9) is a derivative of pMP92 containing the origin of replication, origin of transfer and tetracycline resistance gene of the $IncP_2$ plasmid RK2, as well as the *lacZ*α region of pUC119. Plasmid pCPP30 was used successfully in the past for the expression of the *Pseudomonas syringae* ice nucleation gene *inaZ* in *X. campestris* [9].

During the process of cloning, *E. coli* DH5α was transformed with plasmid pCPP30/P_{wo}*amy* and the transformants were selected for tetracycline resistance and β-galactosidase production. Thirty-nine white Tc^r, were selected on X-gal plates and their plasmids were digested with *Pst*I. Four transformants were identified to contain a 8.9-kb plasmid, with pCPP30 (7.5-kb) and *P. woesei* α-amylase (1.4-kb) DNA sequences as confirmed by restriction and DNA hybridization analysis. Among them, one transformant was subjected to diagnostic restriction analyses, using the restriction endonucleases *Pst*I or *Eco*RI and *Mlu*I, which confirmed the appropriate orientation of the P_{wo}*amy* fragment on plasmid pCPP30/P_{wo}*amy* in frame with the control of the P*lac* promoter.

Plasmid pCPP30/P_{wo}*amy* was subsequently transferred in *X. campestris* ATCC 13951 by electroporation. Transformants were obtained at a frequency of 10^5/μg DNA. Six of them were selected, grown and verified to harbor DNA pCPP30/P_{wo}*amy* by plasmid DNA isolation, restriction and Southern hybridization. These transformants were assayed for α-amylase activity at 95 °C and pH 5.5. *E. coli* DH5α cells harboring *amy* plasmids were also tested as positive controls.

Two out of six colonies exhibited α-amylase activity comparable to that of the *E. coli* recombinants. Lactose and IPTG did not influence the enzyme activity in *X. campestris* cells, implying that *P. woesei* gene may have been expressed by an additional upstream, uncharacterized promoter, or that the culture medium already contains traces of component (s) serving as inducer(s) (Fig. 2). *X. campestris* or *E. coli* harboring pCPP30 did not show any α-amylase activity at 95°C (data not shown) when the strains were grown on LBG or LB, respectively.

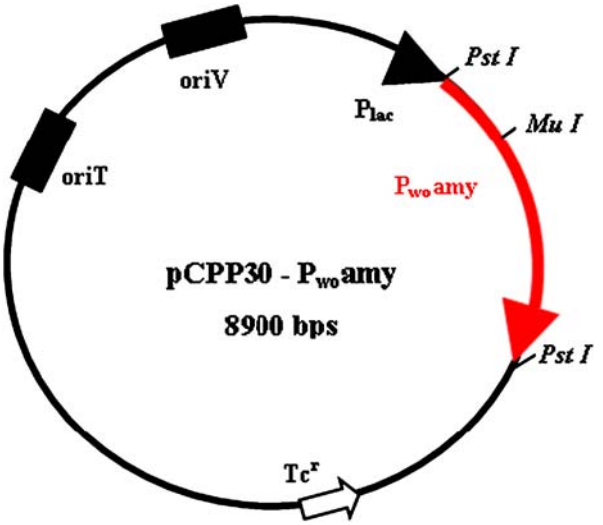

Fig. 1 Map of plasmid pCPP30/P_{wo}*amy*. Plasmid pCPP30/P_{wo}*amy* contains the tetracycline resistance determinant (*Tc*^{r}), origin of transfer (*oriT*), origin of vegetative replication (*oriV*), α-amylase gene of *P. woesei* (P_{wo}*amy*) and the promoter of lactose operon (P_{lac})

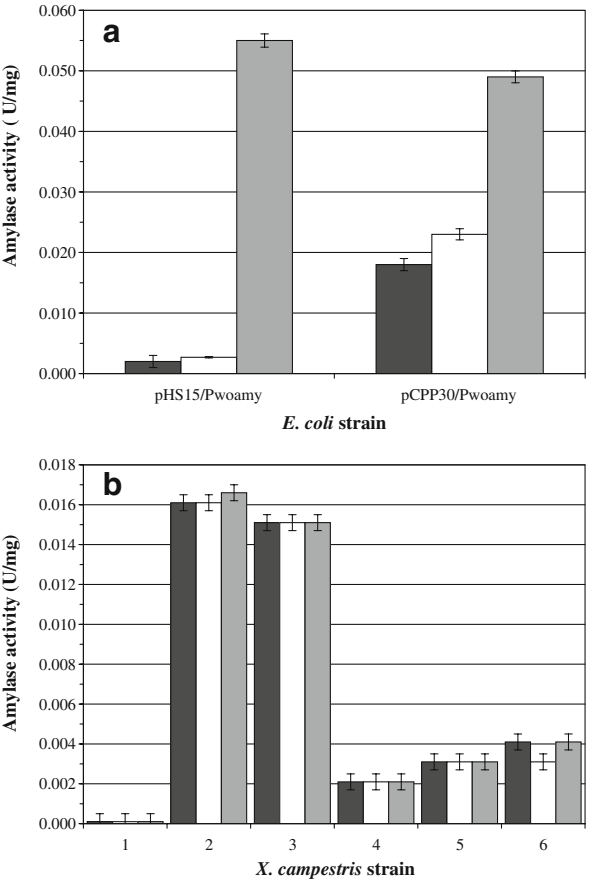

Fig. 2 Intracellular α-amylase activity in *E. coli* and *X. campestris* harboring *amy* plasmids. **a** α-Amylase production by *E. coli* DH5α cells harboring the pHS15/P_{wo}*amy* or the pCPP30/P_{wo}*amy* plasmid grown in LB medium (*filled graph*) and LB medium supplemented with 25°mM lactose (*empty graph*) or 1°mM IPTG (*gray shaded graph*). **b** α-Amylase production by the six colonies of *X. campestris* ATCC13951 cells harboring the pCPP30/P_{wo}*amy* plasmid grown in a LBG medium (*filled graph*) and LBG medium supplemented with 25°mM lactose (*empty graph*) or 1°mM IPTG (*gray shaded graph*)

Plasmid pCPP30/P_{wo}*amy* isolated from the two *X. campestris* transformants was structurally stable. The segregational stability of the plasmid was 80% after 50 cell divisions under nonselective conditions and over 90% after 100 cell divisions under selective conditions. Although the native *P. woesei* α-amylase is extracellular [14], the hyperthermophilic activity detected in *X. campestris* transformants was located only in the crude cell-free extract, as in the case reported for *H. elongata* and *E. coli* cells harboring the α-amylase gene of *P. woesei* [7]. No activity was found in the culture supernatant.

The Influence of Carbon Source on Recombinant and Native α-Amylase Activity

Based on the analysis of α-amylase hyperthermophilic activity, the expression profiles of the native and heterologously expressed *P. woesei* α-amylase were further studied by growing *X. campestris* transformants in minimal M9 medium supplemented with 0.1–2% (*w*/*v*) glucose or starch. Similar levels of recombinant α-amylase activity were detected when *X. campestris* transformants were grown in LBG or the minimal M9 medium supplemented with glucose or starch (Figs. 2 and 3). On the contrary, the production of native α-amylase by *X. campestris* appeared to be dependent on starch availability since no

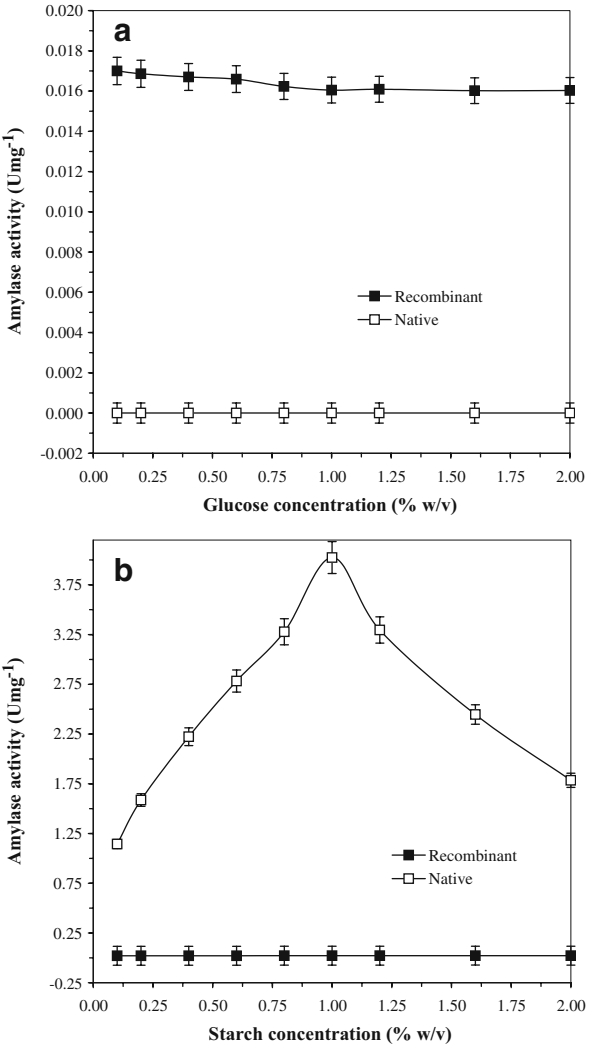

Fig. 3 Effect of glucose (**a**) or starch (**b**) concentration on the expression of recombinant (intracellular, *filled square*) and native (extracellular, *empty square*) α-amylase activity

amylolytic activity was detected when the bacterium was grown on glucose, other reducing sugars or LBG. The highest native α-amylase yield was obtained in the presence of 1% (*w*/*v*) starch reaching approximately 4 U mg^{-1}. Further increase of the substrate concentration resulted in gradual reduction of the enzyme production, which was restricted to 1.8 U mg^{-1} when starch concentration was elevated to 2% (*w*/*v*). The observed relationship between starch concentration and production of native α-amylase by *X. campestris* may be due to the repression caused by starch-hydrolysis products such as glucose. Although the recombinant α-amylase activity was lower in comparison to the native *X. campestris* α-amylase (Fig. 3), the potential application of an amylolytic enzyme in an industrial starch

hydrolysis process is highly dependent on its properties and thus the recombinant and natural α-amylase should be evaluated as well, in terms of their thermostability.

Comparison of the Thermostability of Native *X. Campestris* α-Amylase and Recombinant Enzyme

The recombinant α-amylase was active from 50°C to at least 145°C with a temperature optimum at 95–100°C (Fig. 4), as described before for the native pyrococcal α-amylase. The natural enzyme, on the other hand, demonstrated significantly lower thermophilicity since its highest activity occurred around 60 °C. From 20 to 60 °C the natural α-amylase displayed a steep rise of activity while it declined sharply above 80 °C.

Thermal stability of the native and the recombinant α-amylase was studied in the temperature range 40–70 °C and 100–120 °C, respectively (Fig. 5). The recombinant α-amylase exhibited remarkably higher thermostability in comparison to the native *X. campestris* enzyme. The α-amylase produced by *X. campestris* transformants was completely stable at 90 °C (data not shown) and retained 85% of residual activity at 100 °C after 4 h incubation (Fig. 5). More than 50% of its initial activity was retained after 4 and 0.75 h of incubation at 110 and 120 °C, respectively. The native *X. campestris* enzyme showed a half-life of 4 and 2 h at 40 and 50 °C, respectively, while more than 60% of its activity was lost within the first 30 min when the temperature was increased to 70 °C. Higher rates of inactivation were observed upon further temperature increase (data not shown).

Evaluation of Optimum Conditions for Xanthan Gum Production and Expression of Hyperthermophilic of α-Amylase Activity

It is well known that growth of *X. campestris* and xanthan formation have different nutrient requirements, xanthan biosynthesis being favored by high concentrations of carbon, while there must be enough nitrogen to support growth [15, 16]. In the present study, xanthan gum production by both (wild-type and recombinant) *X. campestris* strain was favored by high concentration of sugars, with glucose appearing to be the most efficient carbon source

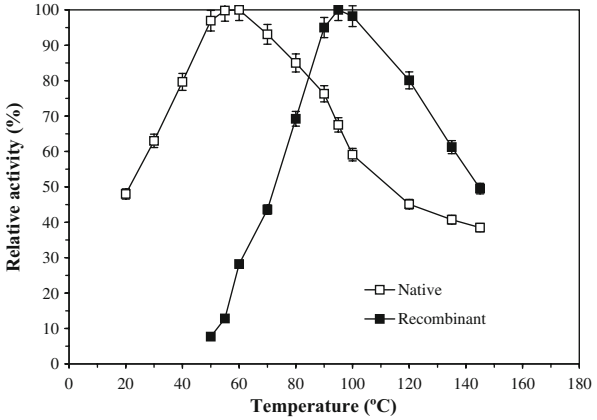

Fig. 4 Effect of temperature on the activity of the recombinant (intracellular, *filled square*) and native (extracellular, *empty square*) α-amylase produced by *X. campestris*

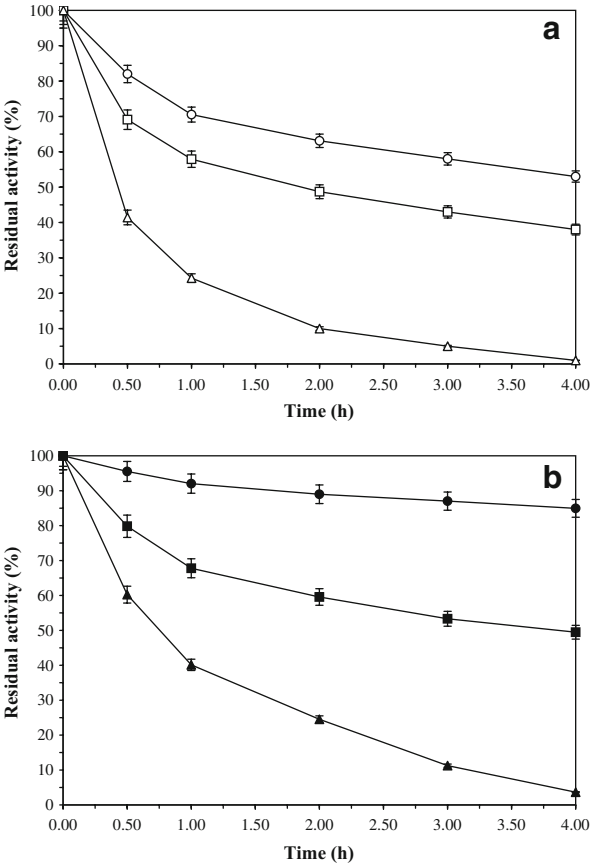

Fig. 5 Comparison of the thermostability of the native (extracellular) and the recombinant (intracellular) α-amylase produced by *X. campestris*. **a** Thermostability of the native (extracellular) α-amylase at 40°C (*empty circle*), 50°C (*empty square*), and 70°C (*empty triangle*). **b** Thermostability of the recombinant (intracellular) α-amylase at 100°C (*filled circle*), 110°C (*filled square*), and 120°C (*filled triangle*)

(data not shown). Under these conditions, the native α-amylase activity is minimal, whereas the recombinant (hyperthermophilic) enzyme is not affected. On the other hand, at 0.6% (*w*/*v*) starch, where native α-amylase activity is maximum, xanthan gum production is impaired (Fig. 6). Increase of starch concentration from 0.6 to 2% (*w*/*v*), which reduces native α-amylase activity as shown above, resulted in twofold higher production of xanthan gum (Fig. 6). In all cases, the activity of the recombinant α-amylase was expressed constitutively at similar high levels. These findings indicate the potential of *X. campestris* cells harboring plasmid pCPP30/P_{wo}*amy* to co-produce two industrially valuable products, such as thermostable α-amylase and xanthan gum, under the same culture conditions and imply a superiority of the transformant cells against their wild-type counterparts.

Discussion

In the last decade, a number of thermophilic microorganisms were found to produce extracellular enzymes that are capable of degrading starch. Since these thermophilic isolates are not considered suitable for use in commercial processes because of their very low

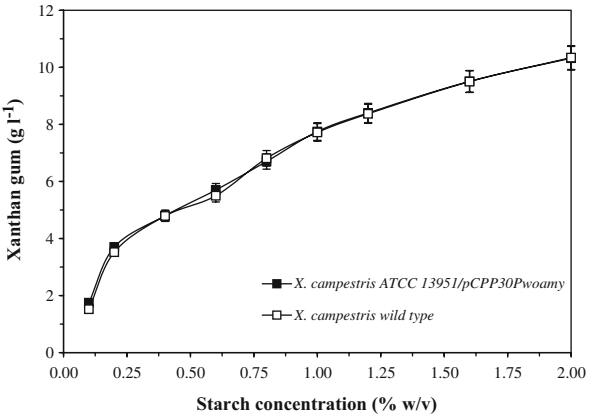

Fig. 6 Effect of starch concentration on xanthan gum production from *X. campestris* transformants (*filled square*) or *X. campestris* wild type (*empty square*)

productivity and tedious cultivation, it is imperative to express the thermophilic genes in mesophilic hosts [3, 4, 17]. *E. coli* has been utilized for many years as the organism of choice for the production of genetically engineered protein products. However, significant problems have arisen in practice; cytoplasmic overexpression of foreign proteins has frequently resulted in insoluble and inactive aggregates [18].

Recently, a *P. woesei* α-amylase was cloned and sequenced and its gene was expressed in *E. coli* and *H. elongata* [7]. In the present work, we demonstrate that the archeal α-amylase was expressed in *X. campestris* using the novel expression vector pCPP30. To our knowledge, this is the first report on the successful expression of an archeal amylase in a *X. campestris* strain. The production of thermostable α-amylase by the mesophilic host *X. campestris* was achieved at about 70 °C below the optimum growth temperature of *P. woesei*, thus demonstrating that high temperatures are not necessary for the correct folding of the protein and that the enzyme does not require the presence of additional extrinsic factors to acquire its thermophilicity and thermostability. The results show that the recombinant *P. woesei* α-amylase not only can be expressed in an active form in *X. campestris* but also that *X. campestris* can substitute *E. coli* as a host, since equivalent activity and thermostability profiles were observed in the two cases.

The heterologously expressed α-amylase in *X. campestris* exhibited catalytic properties matching those of the parent *P. woesei* α-amylase. The optimal activity of the recombinant enzyme was found at 95–100 °C and pH of 5.5–6.0. This high thermostability of the pyrococcal α-amylase renders the recombinant enzyme superior to the native *X. campestris* α-amylase and makes it an interesting candidate for industrial applications. Although *P. woesei* α-amylase is extracellular [7], the recombinant *P. woesei* α-amylase expressed in *X. campestris* was recovered in the crude intracellular fraction. The factors necessary for the secretion, as well as, for the proper folding of the protein need to be investigated for the *X. campestris* host.

In addition, the present study indicates that it is possible to produce two industrially interesting products in one procedure. The ability of *X. campestris* transformed strain to express thermostable α-amylase at high carbon concentration, conditions under which xanthan biosynthesis is favored, not only constitutes the transformants superior to the wild-type strain but it also provides a valuable perspective for this strain in biotechnology. Moreover, the ability of *X. campestris* transformants to utilize industrial by-products, such as molasses, cheese whey, and corn syrup [9, 13, 15], for the co-production of xanthan gum

and α-amylase confer a yet another attractive trait for a low-cost large scale production of the two valuable products.

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References

1. Sanford, P. A., & Baird, J. (1983). In G. O. Aspinal (Ed.) *The polysaccharides, vol. 2* p. 411. Toronto: Academic.
2. Hu, N. T., Hung, M. N., Huang, A. M., Tsai, H. F., Yang, B. Y., Chow, T. Y., et al. (1992). *Journal of General Microbiology*, *138*, 1647–1655.
3. Lévêque, E., Janecek, S., & Belarbi, H. B. (2000). *Enzyme and Microbial Technology*, *26*, 3–14.
4. Dong, G., Vieille, G., & Zeikus, J. G. (1997). *Applied and Environmental Microbiology*, *63*, 3569–3576.
5. Konsula, Z., & Liakopoulou-Kyriakides, M. (2004). *Process Biochemistry*, *39*, 1745–1749.
6. Janecek, S. (1994). *European Journal of Biochemistry*, *224*, 519–524.
7. Frillingos, S., Linden, A., Niehaus, F., Vargas, C., Nieto, J. J., Ventosa, A., et al. (2000). *Journal of Applied Microbiology*, *88*, 495–503.
8. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). In *A laboratory manual, vol. 2: Molecular cloning, 2nd edn*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
9. Afendra, A. S., Yiannaki, E. E., Palaiomylitou, M. A., Kyriakidis, D. A., & Drainas, C. (2002). *Biotechnology Letters*, *24*, 579–583.
10. White, T. J., & Gonzalez, C. F. (1995). In J. A. Nickoloff (Ed.) *Methods in molecular biology, vol. 47: Electroporation of Xanthomonas* pp. 135–141. Totowa, NJ USA: Humana Press.
11. Afendra, A. S., Vargas, C., Nieto, J. J., & Drainas, C. (2004). In J. A. Nickoloff (Ed.) *Methods in molecular biology, vol. 67: Gene transfer and expression of recombinant proteins in moderately halophilic bacteria* pp. 209–223. Totowa, NJ USA: Humana Press.
12. Bradford, M. M. (1976). *Analytical Biochemistry*, *78*, 248–254.
13. Liakopoulou-Kyriakides, M., Psomas, S. K., & Kyriakidis, D. A. (1999). *Applied Biochemistry and Biotechnology*, *82*, 175–180.
14. Koch, R., Spreinat, A., Lemke, K., & Antranikian, G. (1991). *Archives in Microbiology*, *155*, 572–578.
15. Moreno, J., Lopez, M. J., Vargas-Garcia, C., & Vazquez, R. (1998). *Journal of Industrial Microbiology & Biotechnology*, *21*, 242–246.
16. Lo, Y. M., Yang, S. T., & Min, D. B. (1997). *Applied Microbiology and Biotechnology*, *47*, 689–694.
17. Sidhu, G. S., Sharma, P., Chakrabarti, T., & Gupta, J. K. (1997). *Enzyme and Microbial Technology*, *21*, 525–530.
18. Oriel, P., & Schwacha, A. (1988). *Enzyme Microbiology Biotechnology*, *10*, 42–46.